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An endothelin-1 mediated autocrine growth loop involved in human renal tubular regeneration

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An endothelin-1 mediated autocrine growth loop involved in human renal tubular regeneration. Renal tubules have the capacity to regenerate following injury. We have investigated the possibility that tubular-derived endothelins, acting as autocrine growth factors, may be involved in this response in human kidney. ET-1 immunoreactivity was demonstrated by immunohistochemical staining in proximal tubules, distal cortical tubules and medullary collecting ducts of human kidney. In cultured human renal proximal tubular cells, RNAase protection assays demonstrated the expression of ET-1 and ET-2 mRNA's, and radioimmunoassay, following separation of conditioned medium by reverse phase HPLC, showed immunoreactive material which co-eluted with ET-1 and ET-2. Competition binding studies revealed the presence of at least two types of endothelin receptor: one with high and one with low affinity for ET-3 relative to ET-1. Analysis of cellular RNA by RT-PCR demonstrated expression of mRNA's for both ET_A and ET_B receptor subtypes. Combined blockade of ET_A and ET_B receptors (by PD-145065) but not that of ET_A receptors alone (by BQ-123) blocked the mitogenic effect of exogenous or endogenous ET-1 and also profoundly suppressed endogenous ET-1 synthesis. By contrast, incubation with the ET_B receptor agonist, BQ-3020, stimulated endogenous ET-1 synthesis. Exposure of the cells to hypoxia (1% O_2 for 16 to 24 hr) resulted in specific up-regulation of ET-1 but not ET-2 gene expression. These findings reveal the existence of a hypoxia-inducible, autocrine growth system in human proximal tubular cells, which is mediated by ET-1 through the ET_B receptor, and which could function *in vivo* as an autoregenerative system for restoring tubular integrity after injury. The widespread distribution of ET-1 peptide in different tubular segments suggests that ET-1 mediated tubular regeneration may also occur in other nephron segments.

Acute renal failure most commonly arises in the context of hemodynamic disturbance and is associated with ischemic necrosis of renal tubules [1]. Since full renal recovery does occur in the vast majority of cases, the capacity of tubular cells, especially proximal tubular cells, which arguably bear the brunt of the ischemic process [2], to repair and regenerate themselves must be considerable. Although the process of tubular regeneration remains poorly understood, it seems likely that a multiplicity of growth systems, such as endocrine, paracrine and autocrine, are involved in renal tubular regeneration [1]. Several well-characterized polypeptide growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor-I (IGF-I) are normally synthesized in the kidney [3]. There is indirect evidence implicating these growth factors in the process of renal regeneration [4–9]. It is therefore tempting to speculate that renal regeneration following ischemia results from an increase in endogenous renal synthesis of polypeptide growth factors. Direct evidence of this has, however, been conflicting, since renal growth factor gene expression in the ischemic kidney may increase such as HGF [10] or decrease such as EGF [9] and IGF-I [8]. It is also unlikely that such factors operate in all tubular segments, since growth factor synthesis in the normal and ischemic kidney remain restricted to specific tubular segments [3, 11].

The endothelins are a family of novel peptides with particularly potent vasoactive [12] and mitogenic actions [13]. Endothelin, since renamed ET-1, was initially isolated from conditioned media of porcine aortic endothelial cells [14]. Subsequent screening of a human cDNA library by low stringency hybridization revealed the existence of three separate human endothelin genes encoding three related peptides: ET-1, ET-2 and ET-3 [12]. Examination of other species has shown striking conservation of the three endothelin genes throughout evolution, suggesting that they encode peptides with important biological functions. Multiple non-endothelial cell types in a variety of organs including mesangial cells [15] and renal tubular epithelial cells [16] appear to bind and/or synthesize endothelins, making it seem likely that the endothelins have a variety of non-cardiovascular functions, including the regulation of sodium transport [17, 18], cell growth [13, 19] and differentiation during embryonic development [20].

Several observations suggested to us that tubular-derived ET-1 (probably the main contributor to total renal ET-1 synthesis) might play a role in the regenerative process in human renal proximal tubules following ischemia, possibly as an autocrine growth factor which could be up-regulated by cellular hypoxia. First, the level of ET-1 mRNA in the rat kidney is markedly increased during the period of recovery which follows removal of a clamp on the renal artery, and the increase persists for up to seven days [21]; second, hypoxia is known to up-regulate ET-1 synthesis *in vitro* in human umbilical vein endothelial cells (HUVEC) [22]; third, ET-1 is a potent mitogen in cell types other than tubular cells [23]. Because multiple renal cells are known to synthesize ET-1 constitutively, we have investigated this hypothesis using an *in vitro* cell culture model of human renal proximal tubular cells.

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Methods

Immunohistochemical localization of endothelin in human kidney

Immunohistochemical staining for ET-1 in human renal biopsy tissue was performed on renal tissue obtained from ten patients with normal renal function (median plasma creatinine 90 μmol/liter) biopsied because of microscopic hematuria. Histologically, all the biopsies were normal in appearance on light microscopy. A diagnosis of thin membrane disease was made in six patients based on electron microscopy; no definite cause for the hematuria was found in the remaining four patients. The median age was 52 years and male:female ratio was 4:6. Tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Three to 5 μm sections were transferred onto silanized glass slides, dried at room temperature and then for 30 minutes at 56°C. Sections were deparaffinized through xylene, graded concentrations of ethanol and distilled water. Endogenous peroxidase was blocked with 0.5% hydrogen peroxide dissolved in methanol for up to 15 minutes. A high affinity ($K = 4.4 \times 10^{10}$ liter/M) monoclonal antibody, with equal cross reactivity to ET-1, and Big ET-1, which we have generated and described previously [24], was used as the primary antibody. Tissue sections were incubated for 30 to 45 minutes with various dilutions of the primary antibody. A biotinylated rabbit anti-mouse antibody (1/20 dilution) was used as the secondary antibody, and normal human serum was added simultaneously to reduce non-specific binding. Binding of the secondary antibody was detected with a streptavidin-ABC complex linked to horseradish peroxidase, developed with 3'3-diaminobenzidine (DAB) solution, and the sections counterstained with hematoxylin. Control sections were stained, omitting either primary or secondary antibodies or using equivalent concentrations of mouse ascites as the primary antibody. The strength of ET-1 immunoreactivity in three tubular segments, that is, proximal tubules ($N = 8$), cortical distal tubules ($N = 8$) and inner medullary collecting ducts ($N = 4$) was compared by densitometric analysis using the COLORMORPH program (Perspective Instruments Ltd., Essex, UK). Where sufficient tissue was available for analysis, at least 5 high power fields per section, chosen at random, were quantified to obtain a mean value for each tubular segment.

Culture and characterization of human proximal tubular cells

To investigate the regulation of endothelin synthesis in human tubular cells, human renal cortical epithelial cells, derived from the unaffected pole of renal cortex obtained from nephrectomy specimens removed for malignancy or from cadaveric kidneys (considered unsuitable for transplantation for technical reasons), were cultured on collagen-coated flasks as previously described [24–26]. Cells were grown in DMEM/F12 supplemented with triiodothyronine (4 pg/ml), hydrocortisone (36 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) and epidermal growth factor (10 ng/ml) [27]. Cultured cells demonstrated characteristics of a highly enriched population of proximal tubular cells as previously described [24–26]. Contamination by endothelial cells was excluded by uniform negative staining for Von Willebrand factor. At early passages, dome formation was observed, indicating vectorial fluid transport. The cells formed tubular structures with lumens in a three-dimensional Type I collagen gel even at late passage, indicating that they retained polarity. Cyclic AMP production in these cells was up-regulated by parathyroid hormone (PTH, 1 μM) but not by arginine-vasopressin (AVP, 1 μM) in the presence of 3-isobutyl-methyl-xanthine (IBMX) (data not shown). Only cells up to passage 5 were used.

Separation of endothelin peptides in conditioned medium by reverse-phase high performance liquid chromatography (RP-HPLC)

Conditioned medium from confluent monolayers of human PTC (passages 2 to 4) grown in serum-free defined medium was pooled and frozen at −70°C. After thawing, 500 ml of pooled supernatant was acidified with acetic acid (100%) and concentrated through a Sep-pak C18 column as described by Kosaka et al [28]. Material retained on the column was eluted in 4 ml of an ethanol (86%), acetic acid (4%) and water (10%) mixture. The eluate was dried under nitrogen, redissolved in 1 ml of ethanol and dried again. Immediately prior to chromatography on a C18 column (Aquapore OD300, 4.6 × 100 mm; Applied Biosystems Inc., San Jose, CA, USA), the preparation was redissolved in 100 μl of acetonitrile (100%) containing 0.1% trifluoroacetic acid. Using a flow rate of 0.5 ml/min, the gradient of acetonitrile was increased linearly from 0 to 20% over five minutes and 20 to 40% over 30 minutes. Preliminary work had shown that this gradient provided clear separation of standards (1 nmol) of ET-1, ET-2, ET-3 and ET-1 sulphoxide. ET-1 sulphoxide was generated by oxidation of ET-1 with 0.5% hydrogen peroxide in 1 M formic acid at 30°C for six hours [29]. 0.25 ml fractions were collected every 0.5 minute, dried in a vacuum centrifuge and kept at −70°C until assayed. The recovery of ET-1 standard using this procedure was 90%.

Endothelin immunoassays

Cell supernatants were assayed for immunoreactive endothelin (IR-ET) by an immunoradiometric assay (IRMA) specific for ET-1 developed by us and reported previously [22, 24]. The assay showed a 15% cross reaction with ET-2 and ET-3 throughout the dose response curve, no cross reaction with Big ET-1 up to a level of 500 ng/liter of sample and no cross reaction with human atrial natriuretic peptide (<0.0001%). In addition, there was no cross-reactivity with the ET analogues, BQ-123 (10^{-6} M), BQ-3020 (10^{-7} M) and PD-145065 (10^{-5} M) at the concentrations used. The sensitivity of the assay (3 × SD of zero dose estimate) was 0.15 pg/tube, corresponding to 1.5 pg/ml in cell supernatants. Inter- and intra-assay variations were 10% and 4.7%, respectively.

Fractionated samples from HPLC were also assayed using a commercially available radioimmunoassay (RIA) (Nichols Institute Diagnostics, Wijchen, The Netherlands). The antibody used in this assay cross-reacts with ET-1 (100%), ET-2 (52%), ET-3 (96%) and Big ET-1 (7%). The sensitivity of the assay is 2 pg/ml.

RNAase protection assays for human ET-1, ET-2 and ET-3 mRNAs

RNA extraction from human PTC was performed using a one-step procedure (RNAzol™B, Biogenesis Ltd, Bournemouth, UK) [30] according to the manufacturer's instructions. Riboprobes specific for human ET-1, ET-2 and ET-3 mRNAs were generated as previously described [31]. In brief, two-stage polymerase chain reactions (PCR) using partially nested primers designed from known gene sequences were employed to amplify

short genomic fragments of ET-1, ET-2 and ET-3; in each case including part of the coding sequence from exon 2 (which encodes the amino acids present in the mature peptides) together with adjacent 5′ intron. The fragments were cloned into appropriate riboprobe vectors, linearized and used to produce specific labeled antisense RNA transcripts by *in vitro* transcription with SP6 polymerase in the presence of [α-^{32}P]GTP (Amersham International, Amersham, UK). For analysis of mRNA, precipitated total RNA was dissolved in an aliquot of hybridization buffer (80% formamide, 40 mM PIPES, 400 mM sodium chloride, 1 mM EDTA pH 8) and the RNA concentration determined by absorbance measurements at 260 nm using a DU-62 spectrophotometer (Beckman Instruments Inc). The concentrations were adjusted to yield 50 μl samples containing 30 μg of RNA, to each of which was added 0.2 μg of total RNA extracted from rat liver. This RNA contained a high level of rat actin mRNA, cohybridization to a rat actin riboprobe [32], providing an internal control to check that RNA degradation, sample processing and gel loading were comparable between specimens. Hybridization was performed overnight at 60°C with 5×10^5 cpm of the appropriate ET probe and 5×10^5 cpm of rat actin probe. RNAase digestion was then carried out at 37°C for 30 minutes, and terminated by the addition of 60 μl of proteinase K (1 mg/ml) with 3% SDS and further incubation at 37°C for 30 minutes. Phenol-chloroform and chloroform extractions were performed and the RNA fragments precipitated with 2.5 volumes of absolute alcohol. The precipitated RNA was dissolved in 5 μl of 80% formamide loading buffer, and the reaction mix electrophoresed on a denaturing 8% polyacrylamide gel. The gel was then dried and subjected to autoradiography at −70°C, following which the protected ET and rat actin mRNA bands were excised and radioactivity counted using a liquid scintillation counter (model 1205 Beta Plate™, Pharmacia-Wallac OY, Turkey, Finland). On a single gel, the counts obtained from the actin bands never varied over a range of more than 15%, indicating that there was no great variation in RNA degradation, processing and gel loading between individual samples. Statistical comparison of ET expression between experimental and control groups was made by comparing counts, normalized for actin recovery, using an unpaired *t*-test with a two-sided *P* value of less than 0.05 being taken as indicating significance.

Effect of hypoxia on ET-1, ET-2 and ET-3 gene expression

Human PTC were grown on 100 mm collagen-coated plates were incubated at 37°C for between 16 to 24 hours under hypoxic conditions by preinfusion of a preanalyzed gas mixture (1% O_2, 5% CO_2, 94% N_2) into an air-tight modular incubator (ICN Biomedicals Ltd., Thame, UK). Medium pO_2 achieved after this period was measured at between 30 to 40 mm Hg using an automated gas analyzer. As normoxic controls, cells were incubated in the same incubator (20% O_2, 5% CO_2) and the medium pO_2 was 140 to 150 mm Hg. In three experiments, cells were also incubated in parallel with cobaltous chloride (50 μM), a compound which mimics the effect of hypoxia in up-regulating the erythropoietin gene [33]. Total cellular RNA was harvested and analyzed by RNAase protection assays for all three endothelin mRNAs as described above. Cell supernatants were saved for ET-1 assay by IRMA.

Reverse transcriptase polymerase chain reaction (PCR) detection of ET_A and ET_B receptor mRNA

First strand cDNA was synthesised from 2.5 μg of total cellular RNA using oligo(DT) primers and avian myoblastosis reverse transcriptase. Nested PCR for ET_A and ET_B receptor mRNA was performed using conditions described in detail in a previous paper [34]. These PCR products had previously been cloned and sequenced on both strands and confirmed as human ET_A and ET_B fragments by comparison with previously published sequences [35, 36]. Internal primer pairs yielded products of 299 base pairs for ET_A and 428 base pairs for ET_B.

Cellular binding of ^{125}I-ET-1

Human PTC were plated in 24-well plates at a density of 1×10^5 cells per well and left overnight. They were then preincubated with binding medium (DMEM/F12 plus 0.1% bovine serum albumin) at 37°C for 30 minutes, before the addition of ^{125}I-ET-1 (Specific activity = 2,000 Ci/mmol, Amersham International) in a volume of 200 μl at different concentrations and for different time periods. Preliminary studies showed that binding of ^{125}I-ET-1 to the cells at 4°C had not reached equilibrium by 24 hours; therefore, all subsequent studies were performed at 37°C. Because of this, B_{max} values quoted may overestimate the total binding capacity of ET-1 since they reflect both binding and uptake of ^{125}I-ET-1.

For saturation binding experiments, cells were incubated with increasing concentrations of ^{125}I-ET-1 for six hours. For competition binding experiments, increasing concentrations of unlabeled ET-1, ET-3 or BQ-123, were also added to binding medium containing ^{125}I-ET-1 (100 pM). At the end of each experiment, cell monolayers were washed extensively with ice-cold binding medium and solubilized with 0.5 N NaOH. Bound and internalised ^{125}I-ET-1 was estimated by gamma counting and total cell protein per well measured by the Bradford method. Binding data were analyzed by data transformation using a modified version of the LIGAND/EBDA program (Elsevier).

DNA synthesis measured by [^{3}H]-thymidine incorporation

Cells were plated in 24-well plates at a density of 1×10^5 cells per well and made quiescent by a 48 hour incubation with basal medium (DMEM/F12) containing 5 μg/ml transferrin. In initial studies, quiescent cells were then incubated for a further 24 hours with different concentrations of ET-1, ET-2 or ET-3. In subsequent experiments, we found the mitogenic effect of exogenous ET-1 to be variable; in some experiments, exogenous ET-1 (10^{-11} M to 10^{-9} M) had no additional mitogenic effect, suggesting that endogenous ET-1 release might be masking this effect. To block the synthesis and action of endogenous ET-1, we preincubated quiescent cells with phosphoramidon (10^{-4} M; which blocks the ET-1 converting enzyme) and the specific endothelin receptor antagonists, BQ-123 (10^{-6} M; which blocks ET_A) and PD-145065 (10^{-5} M; which blocks ET_A and ET_B), respectively. The cells were then pulsed with 1 μCi of [^{3}H]-thymidine per well for 18 to 24 hours. After washing, cells were precipitated with 10% trichloroacetic acid (TCA) and solubilized with 0.5 N NaOH. An aliquot was counted in a liquid scintillation counter and another assayed for total cell protein per well.

To allow for the variability between different experiments performed using cells from different sources, the results are

expressed as percentage increases over control to enable comparison between different experiments to be made.

Effect of ET analogues and phosphoramidon on endogenous ET-1 synthesis

ET-1 synthesis by tubular cells over 24 hours was measured to study the effect of co-incubation with BQ-123, PD-145065, phosphoramidon, and the ET_B receptor agonist, BQ-3020.

Materials

Endothelin standards (ET1, ET2, ET3) and BQ-123 were obtained from the Peptide Institute (Osaka, Japan). PD-145065 was a gift of Dr. Annette Doherty (Parke-Davis, Ann Arbor, MI, USA). BQ-3020 was purchased from Scientific Marketing Associates (Barnet, Herts, UK). Enzymes were obtained from HT Biotechnology (Cambridge, UK) and deoxynucleoside triphosphates from Pharmacia Ltd (Milton Keynes, UK). Except where previously mentioned, all chemicals were obtained from Sigma (Poole, Dorset, UK).

Statistical analyses

All results are expressed as means ± SEM unless otherwise stated. Unpaired Student's *t*-test was used for statistical comparisons and considered significant if the *P* value was less than 0.05.

Results

Localization of ET-1 peptide in human tubular cells in vivo

Immunoreactive ET-1 was demonstrated in the glomeruli, proximal tubules and all tubular segments as well as in the papilla of human kidney (Fig. 1). In the cortex, both proximal tubules and distal tubules stained positively for ET-1, although distal tubules were more strongly positive (Fig. 1A); on some sections at higher magnification (×1000), ET-1 staining in proximal tubular cells appeared to be localized to basal cell inclusions (Fig. 1C). Positive staining was also seen in large vessel endothelium (Fig. 1H) but not in the vasa recta (Fig. 1E). In the medulla, collecting ducts (Fig. 1 D, E) and pelvic epithelium (Fig. 1G) exhibited positive ET-1 immunoreactivity. The specificity of the staining was indicated by the negative controls (Fig. 1 B, F). As assessed by semiquantitative densitometric analysis in 8 specimens (with '0' representing maximal density and '250' representing minimum density), ET-1 staining in three tubular segments (mean ± SE) was highest in the cortical distal tubules (173 ± 5), intermediate in inner medullary collecting ducts (193 ± 4) and lowest in proximal tubular segments (208 ± 2).

Human PTC synthesize ET-1 and ET-2 peptides

Since existing immunoassays cannot distinguish between ET-1 and ET-2, RP-HPLC was used to separate the different endothelin isoforms present in conditioned medium from human PTC prior to immunoassay. Three major immunoreactive peaks, corresponding to ET-1 sulphoxide (retention time 27.5 min), ET-1 (retention time 29.5 min) and ET-2 (retention time 30.5 min), were found in three separate experiments, as assayed by IRMA (Fig. 2). The results by RIA were qualitatively identical, although the peak for ET-2 was higher in line with the greater cross reactivity of the antibody to ET-2 (data not shown). In one experiment, a peak corresponding to ET-3 (retention time 25.5 min) was found (data not shown), but this was not pursued further since ET-3 mRNA was not detected (see below).

Human PTC express ET-1 and ET-2 mRNA, but hypoxia up-regulates ET-1 gene expression only

Both ET-1 and ET-2 mRNA were detected in human PTC in culture (Fig. 3). Exposure of tubular cells to hypoxia (16 to 24 hr) resulted in increased expression of the ET-1 gene but not of the ET-2 gene (1.1 ± 0.1 of normoxic controls, $N = 3$; Fig. 3). Expression of the ET-3 gene was not detected under normoxic or hypoxic conditions in several experiments (data not shown). ET-1 steady-state mRNA levels in hypoxic cells were consistently elevated by 2.6 ± 0.1-fold (mean ± SEM, $N = 7$) after 16 to 24 hours of hypoxia (Fig. 4). ET-1 peptide was correspondingly increased by roughly twofold in supernatants of hypoxic cells (181 ± 17% of normoxic controls, $N = 6$). In three experiments, incubation with cobaltous chloride for 24 hours did not reproduce the effect of hypoxia on ET-1 gene expression (Fig. 4) or peptide synthesis (118 ± 11% of normoxic controls, $N = 3$).

ET_A and ET_B receptor genes are expressed in human PTC

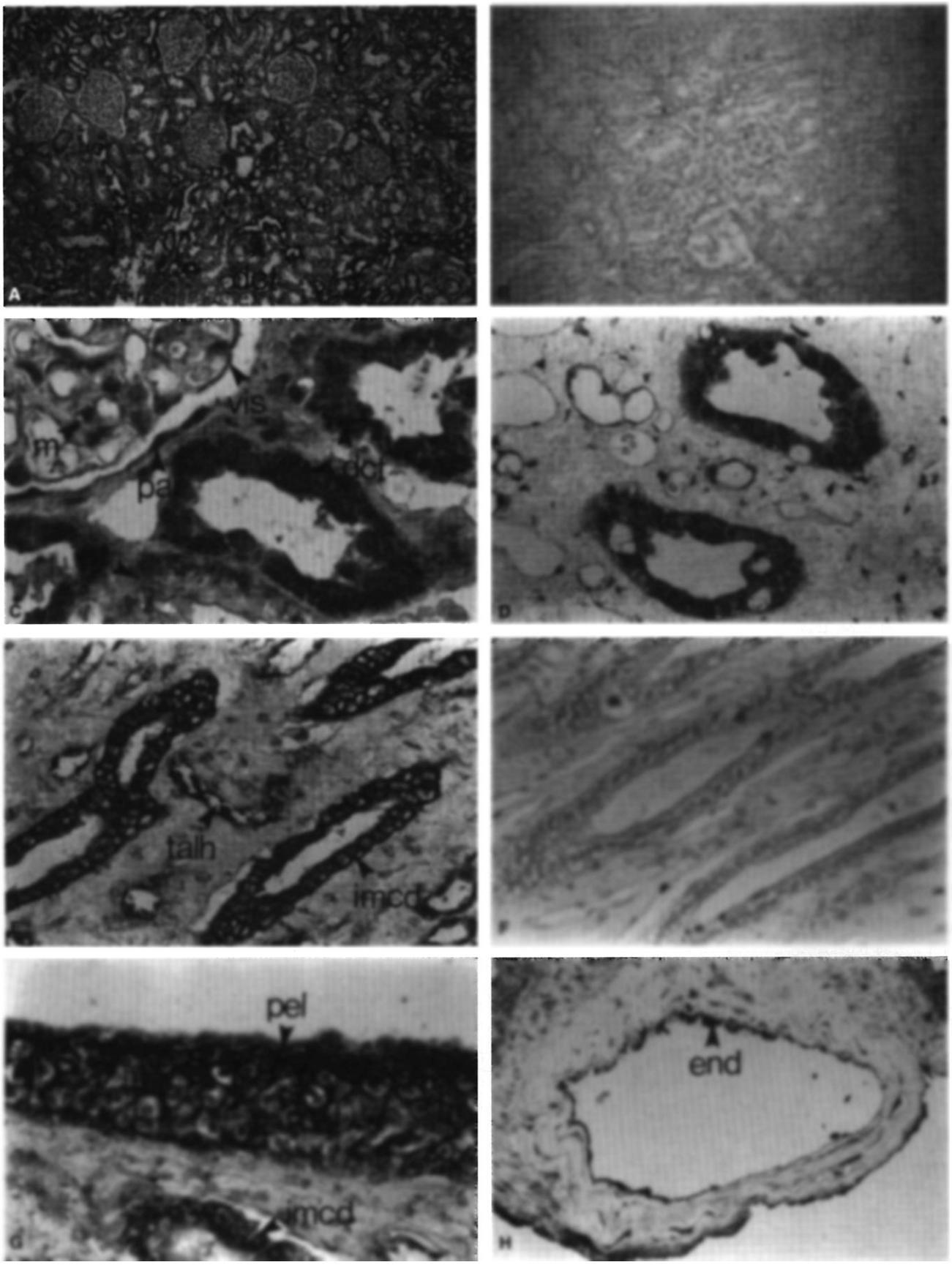
Six separate samples of total RNA extracted from these cells were examined for ET_A and ET_B receptor gene expression. Two bands of 299 base pairs and 428 base pairs, corresponding to the predicted amplified sequences for ET_A and ET_B, were found in all six samples (Fig. 5).

Human PTC bind ^{125}I-ET-1 by two high-affinity receptors

The binding of ^{125}I-ET-1 to tubular cells at 37°C was time-dependent, reaching equilibrium by six hours and remaining constant for a further six hours (data not shown). Scatchard analysis of saturation binding of ^{125}I-ET-1 by PTC revealed a K_d of 41 ± 16 pM ($N = 3$) and a single population of high affffinity receptors (Fig. 6A). Unlabeled ET-1 and ET-3 competed with the binding of ^{125}I-ET-1 with an IC_{50} of 0.33 ± 0.04 nM ($N = 4$) and 320 ± 37 nM ($N = 5$), respectively (Fig. 6B). Transformation of competition binding data between unlabeled ET-1 and labeled ET-1 by LIGAND revealed a K_i of 0.11 ± 0.03 nM ($N = 4$) and a one-site fit. In contrast, data transformation of competition binding between unlabeled ET-3 and labeled ET-1 (Fig. 6B) receptors with low and high affinity for ET-3 relative to ET-1, corresponding to the described features of ET_A and ET_B receptors: calculated values for K_i ($N = 5$) for ET_B were 0.18 ± 0.07 nM and for ET_A, 180 ± 20 nM. B_{max} values for ET_A and ET_B were 8.3 ± 1.3 fmol/μg cell protein and 1.7 ± 0.3 fmol/μg cell protein, respectively; the ratio of ET_A/ET_B was 5.5 ± 1.4 ($N = 5$). These K_d and K_i values closely approximate those described for ^{125}I-ET-1 and ^{125}I-ET-3 binding to cultured rat [37] and porcine [38] tubular cells. The ET_A-specific receptor antagonist BQ-123 competed with ^{125}I-ET-1 for binding with a K_i of 9 ± 1 nM ($N = 6$) and had an IC_{50} of 22 ± 5 nM ($N = 6$), values similar to those described for ET_A receptors expressed by rat vascular smooth muscle cells [39]. B_{max} for ET_A receptors estimated in this way was 7.5 ± 1.6 fmol/μg cell protein.

ET-1 is an autocrine growth factor in human PTC

In initial studies, we found exogenous ET-1, ET-2 and ET-3 to be roughly equipotent in stimulating DNA synthesis in quiescent tubular cells: the maximum increase seen was twofold and the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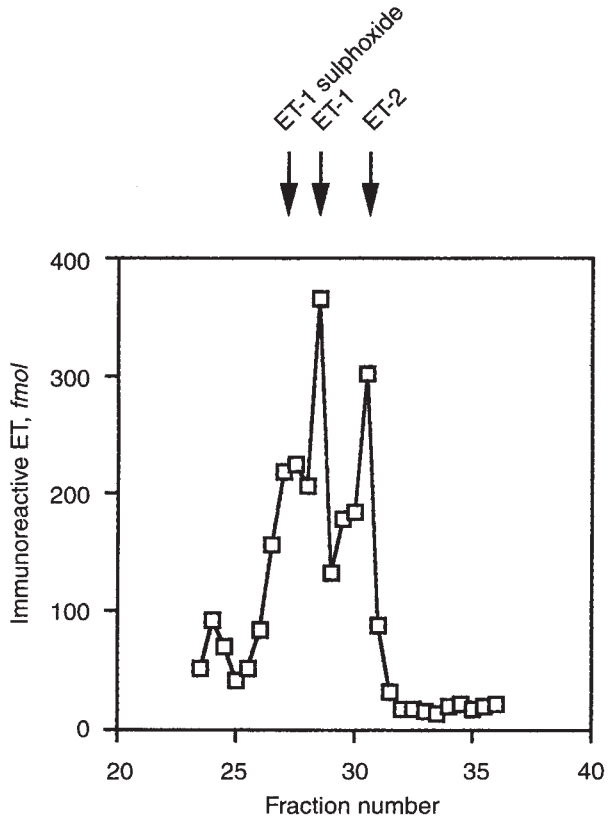


Fig. 2. *Immunoreactive endothelin in conditioned medium of human PTC as assayed by IRMA following separation by reverse-phase HPLC as described in the text.* Three immunoreactive peaks corresponding to ET-1 sulphoxide (retention time 27.5 min), ET-1 (retention time 29.5 min) and ET-2 (retention time 30.5 min) were detected in three separate experiments.

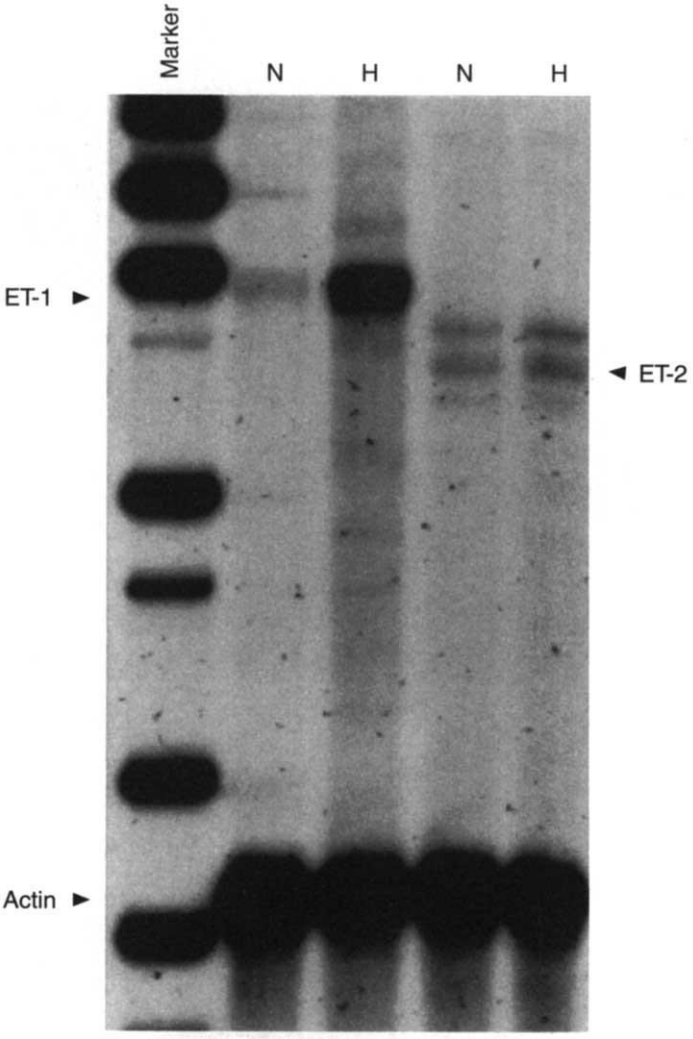


Fig. 3. *Hypoxia up-regulates ET-1 but not ET-2 gene expression in human PTC.* Human PTC were exposed to normoxic (20% O_2) or hypoxic conditions (1% O_2) for 16 hours. Total RNA was extracted and assayed by RNAase protection assays for human ET-1 and ET-2 mRNA as described in the text. ET-3 mRNA could not be detected in normoxic or hypoxic cells (data not shown). These results are representative of three independent experiments. Abbreviations are: N, normoxia; H, hypoxia. Equivalent intensity of actin protected species in all lanes indicates that sample processing and gel loading was comparable for all specimens.

concentration inducing half-maximal response was approximately 10^{-12} M for all three endothelins (Fig. 7A). In addition, the mitogenic effect of exogenous ET-1 could be blocked by PD-145065 (10^{-5} M) but not by BQ-123 (10^{-6} M; data not shown).

To block the effect of endogenous ET-1, cells were incubated with phosphoramidon or PD-145065. Phosphoramidon (10^{-4} M) or PD-145065 (10^{-5} M) both resulted in a 25% decrease in [^{3}H]-thymidine incorporation (Fig. 7B). Co-incubation with both compounds did not decrease basal DNA synthesis further (Fig. 7B). These effects were not related to cell toxicity since there was no significant difference in total cell protein between wells and no evidence of significant cell detachment after incubation (data not shown). Incubation with BQ-123 did not inhibit basal DNA synthesis (Fig. 7B). These results suggest that the ET_B receptor selectively mediates the mitogenic effect of endogenous or exogenous ET-1 in human tubular cells.

Fig. 1. *Immunoreactive endothelin in normal human kidney.* **(A)** Renal cortex (×100) – proximal and distal cortical tubules (dct) stained positively for ET-1; **(B)** renal cortex (×100) – control section with no primary antibody; similar results were seen with equivalent concentrations of normal mouse ascites or when the primary antibody was preincubated with 5 μM ET-1; **(C)** renal cortex (×1000) – weak staining in glomerular parietal epithelium (par), glomerular visceral epithelium (vis) and mesangial cells (m) in addition to distal (dct) and proximal (pt) tubules; **(D)** renal medulla (×200) – positive staining of inner medullary collecting ducts; **(E)** renal medulla (×400) – positive staining of inner medullary collecting ducts (imcd) and thin limbs of ascending loops of Henle (talh); **(F)** renal medulla (×200) – control section stained with primary antibody preincubated with 5 μM ET-1; similar results were seen when equivalent concentrations of mouse ascites were used or when the primary antibody was omitted; **(G)** renal pelvis (×400) – positive staining overlying the multilayered pelvic epithelium (pel); **(H)** renal artery (×400) – positive staining in endothelium (end). The primary antibody used was a monoclonal antibody to human ET-1 with equal cross reactivity to Big ET-1 but less than 10% cross reactivity to human ET-2 and ET-3.

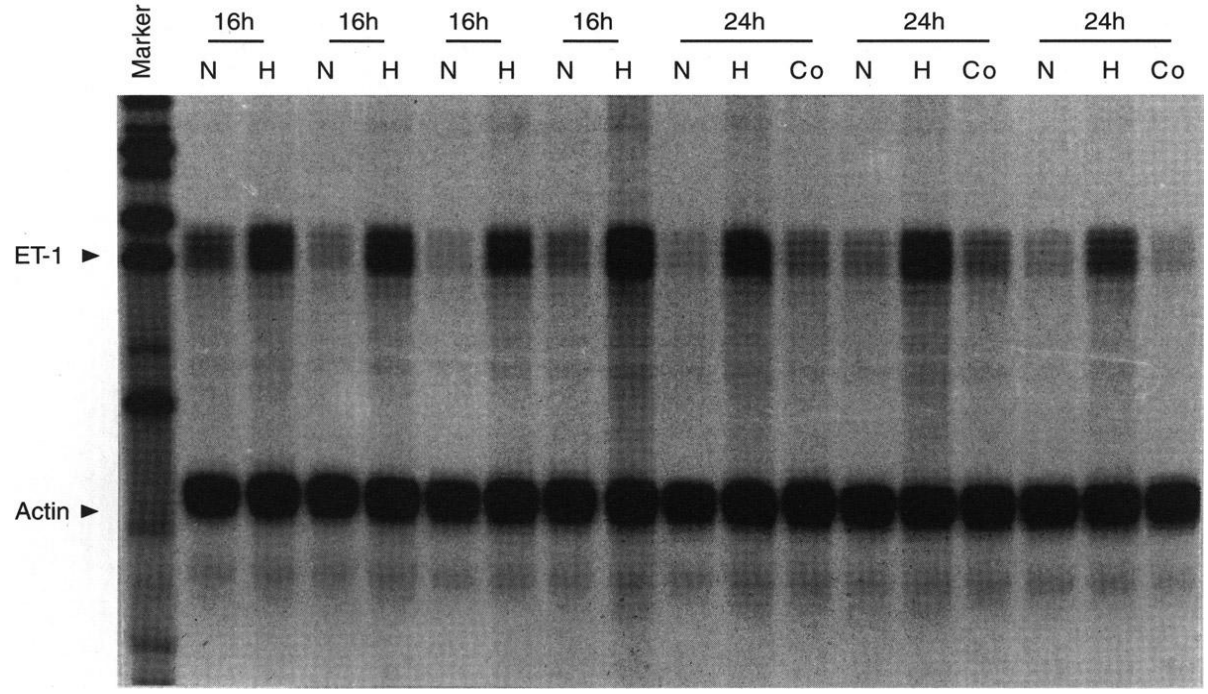


Fig. 4. *Effect of hypoxia on ET-1 gene expression in human PTC is not reproduced by cobalt chloride (Co).* Hypoxia (1% O_2) increased ET-1 mRNA by 2.6 ± 0.1-fold after 16 to 24 hours ($N = 7$). In the latter three experiments, cobaltous chloride (50 μM) had no significant effect on ET-1 gene expression: 1.2 ± 0.1 of control ($N = 3$). Values quoted are means ± SEM. Details as for Figure 3.

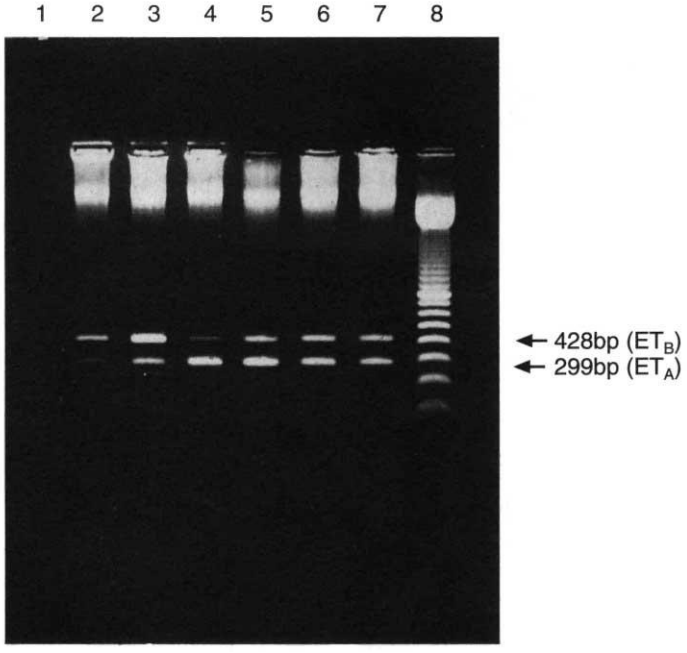


Fig. 5. *Agarose gel showing products of PCR amplification of ET_A (299 bp) and ET_B (428 bp) cDNA from six separate cultures of human PTC (lanes 2 to 7).* Lane 1 is a negative control (no template) and Lane 8 is a 100 bp DNA ladder.

ET-1 stimulates its own synthesis via the ET_B receptor

Incubation with phosphoramidon (10^{-4} M) alone suppressed ET-1 release to 25% of control values over 24 hours (Fig. 8A). Unexpectedly, incubation with PD-145065 (10^{-5} M) was associated with suppression of endogenous ET-1 synthesis to less than 10% of control values (Fig. 8A). In combination, both agents resulted in almost complete suppression (3% control) of endogenous ET-1 production (Fig. 8A). No difference in cell counts or cell protein between wells was found to account for these differences in ET-1 synthesis (data not shown). Also, no significant difference was observed in ET-1 synthesis between controls and cells incubated with BQ-123 (10^{-6} M).

Incubation with the ET_B selective agonist, BQ-3020 (10^{-7} M) [40] stimulated tubular ET-1 synthesis by 43 ± 4% ($N = 10$ to 11) of control cells (Fig. 8B). These results suggest that ET-1 stimulates its own synthesis in human tubular cells by a positive feedback loop mediated by the ET_B receptor.

Discussion

The mechanisms underlying repair and regeneration of renal tubular cells after ischemic acute renal failure remain poorly defined [1]. Clearly, where there is extensive tubular cell death, tubular regeneration from surviving cells must increase in parallel to maintain nephron integrity. How this complex process is initiated is not known, but surviving cells appear to express markers normally associated with dedifferentiation (vimentin expression) and mitogenesis (proliferating nuclear cell antigen, c-*fos* expression) after an ischemic insult [2]. We hypothesized that a major part of this regenerative process could involve hypoxic induction or up-regulation of an autocrine growth factor in surviving cells, leading to appropriate 'autoregeneration' and thus reconstitution of the injured renal tubule.

Because the renal microcirculation is particularly sensitive to the vasoconstrictor action of ET-1 [41], much work has focused on

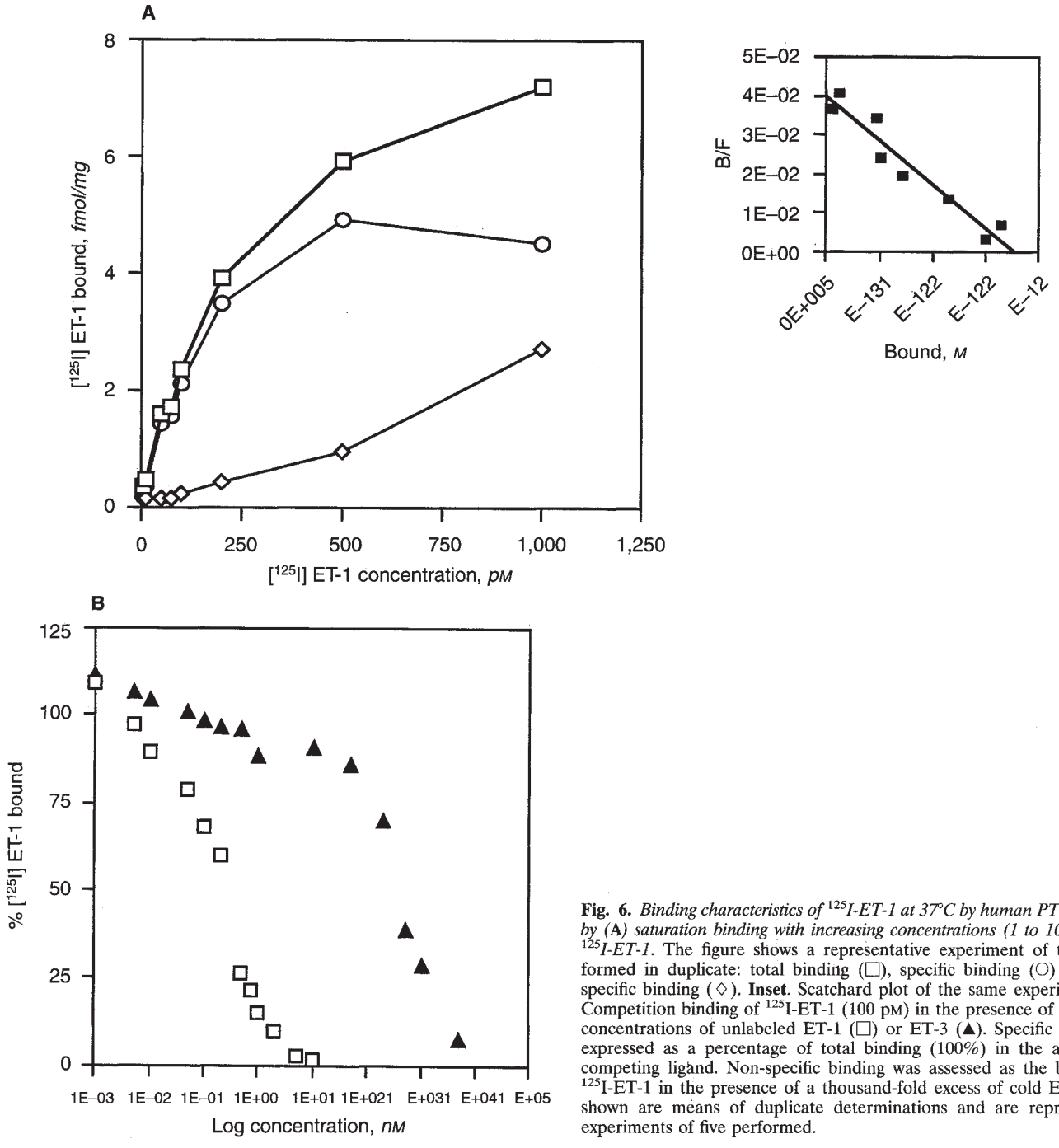


Fig. 6. *Binding characteristics of ^{125}I-ET-1 at 37°C by human PTC assessed by* **(A)** *saturation binding with increasing concentrations (1 to 1000 pM) of ^{125}I-ET-1.* The figure shows a representative experiment of three performed in duplicate: total binding (□), specific binding (○) and non-specific binding (◇). **Inset.** Scatchard plot of the same experiment. **(B)** Competition binding of ^{125}I-ET-1 (100 pM) in the presence of increasing concentrations of unlabeled ET-1 (□) or ET-3 (▲). Specific binding is expressed as a percentage of total binding (100%) in the absence of competing ligand. Non-specific binding was assessed as the binding of ^{125}I-ET-1 in the presence of a thousand-fold excess of cold ET-1. Data shown are means of duplicate determinations and are representative experiments of five performed.

the pathophysiological role of ET-1 in ischemic acute renal failure. The most convincing evidence of such a role comes from studies in rodents where administration of an anti-endothelin antibody, either before [42] or after [43] the onset of renal ischemia, appeared to ameliorate the typical vasoconstriction and histological changes of the post-ischemic kidney. Most attention has thus been directed towards the effects that ET-1 might have on vascular tone or glomerular function. However, several observations suggest that the pathophysiological involvement of ET-1 in these models may not be solely related to vascular or glomerular events. First, no differences in either number or affinity of glomerular endothelin receptors between ischemic and control kidneys were found after a 60-minute ischemic period at a time when glomerular filtration rate (GFR) and glomerular plasma flow (Q_A) were less than 5% of control values [44]. Second, in a model of severe acute renal failure, the ET_A receptor antagonist BQ-123, administered 24 hours after renal ischemia, initiated recovery of tubular function while glomerular filtration remained markedly depressed [45].

Since most of our knowledge of the localization of endothelin

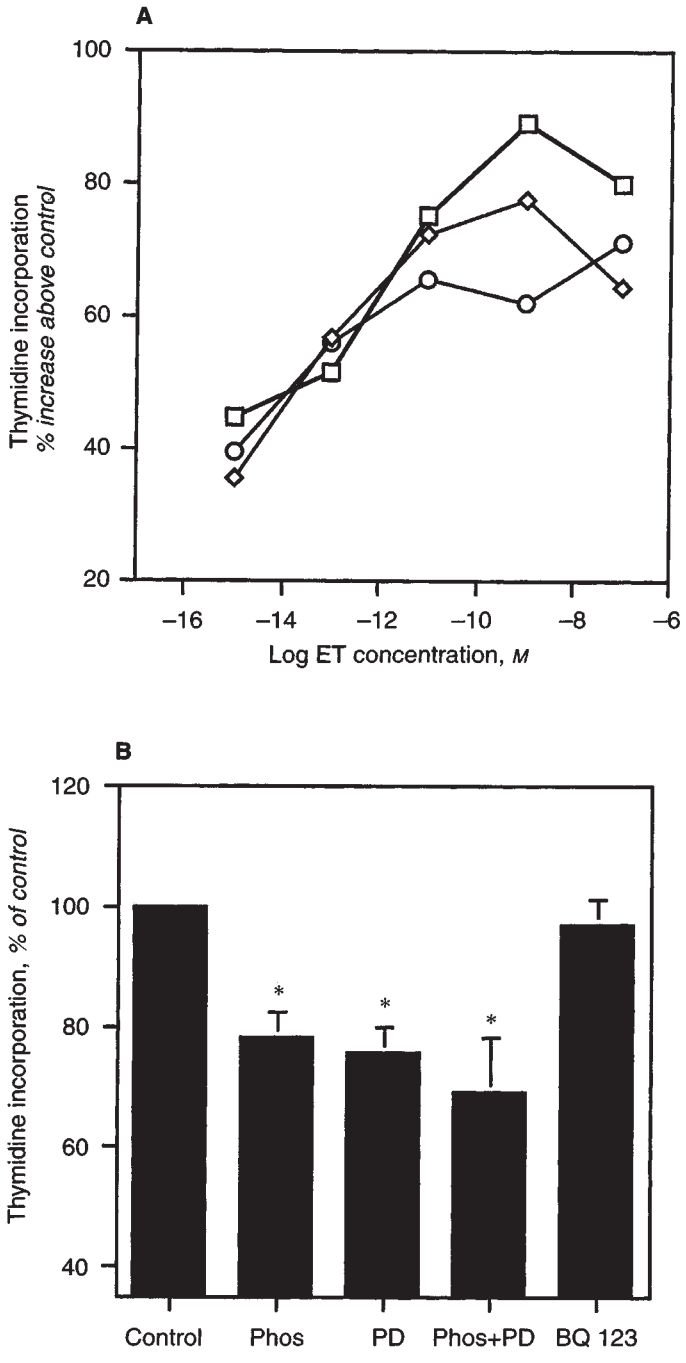


Fig. 7. *Effect of exogenous and endogenous endothelins on DNA synthesis, as assessed by [^{3}H]-thymidine incorporation, in quiescent human PTC.* (**A**) Effect of increasing concentrations (10^{-15} to 10^{-7} M) of exogenous ET-1 (□), ET-2 (◇) and ET-3 (○); the Figure shows mean increases in thymidine incorporation above control of triplicate wells taken from a representative experiment ($N = 2$); error bars have been omitted for the sake of clarity but did not exceed 10% of the mean for each point. Each point was significantly different from controls, but there was no significant difference between ET-1, ET-2 and ET-3 at each concentration tested. (**B**) Effect of phosphoramidon (Phos, 10^{-4} M), BQ-123 (10^{-6} M) and PD-145065 (PD, 10^{-5} M) on basal DNA synthesis; $N = 16$ from four experiments. Results are expressed as a percentage of control (100%) to enable comparison between different experiments; each bar represents mean ± SEM. Exogenous ET-1 (10^{-9} M) elicited no significant increase in [^{3}H]-thymidine incorporation: 101.2 ± 3.3%. * $P < 0.05$ vs. control.

synthesis and its potential functions in the kidney comes from animal studies, in particular from the rat, it should be noted that important species differences between rat, dog and humans have been found. These include the anatomical localization of endothelin receptors [46–48], the isoforms of endothelin found [21] and the endothelin receptor subtype mediating renal vascular responses or tubular sodium excretion [48]. For these reasons, animal models are less than ideal for studying relevant functions of endothelins in the healthy and diseased human kidney. Since the pathophysiology of renal ischemia is complex [49] and multiple renal cells synthesize endothelins [50], we chose an *in vitro* model of human renal proximal tubular cells to investigate the possibility that tubular-derived endothelins might function as autoregenerative factors in human renal proximal tubules following ischemia.

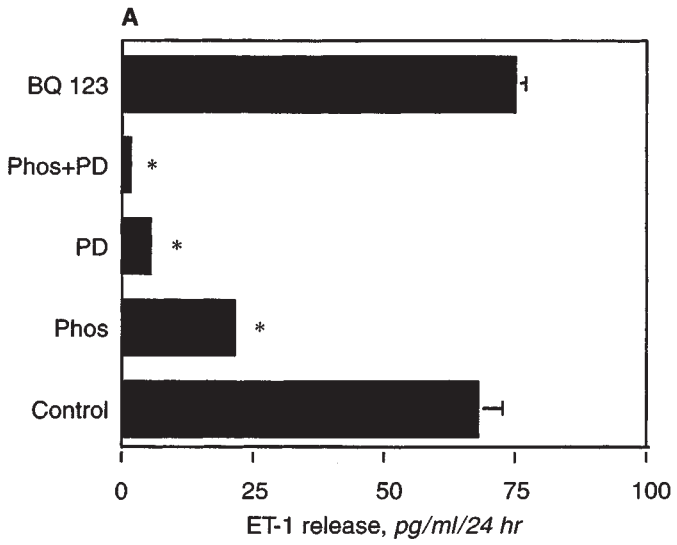
Using immunohistochemical techniques, we were able to localize ET-1 peptide in different segments of the human nephron, including the proximal tubule. Nevertheless, the presence of immunoreactive peptide does not prove *de novo* synthesis. In the absence of *in situ* hybridization studies demonstrating tubular localization of ET-1 mRNA, it is possible that immunoreactive tubular ET-1 staining, especially in the proximal tubule, could represent uptake of filtered ET-1 peptide.

Unlike others [51, 52], we did not find strong staining of the vasa recta or glomeruli. These differences may be either species or technique related. The immunoreactivity of IMCD cells was also less than expected from animal studies [16, 17] since cells from this segment seem to produce the highest ET-1 concentrations *in vitro*. However, a study of ET-1 synthesis by human IMCD cells found much lower ET-1 concentrations than those produced by rat IMCD cells [53]. The significance of papillary staining is unclear.

The RNAase protection assay and RP-HPLC/radioimmunoassay studies showed that cultured human proximal tubular cells express ET-1 and ET-2 mRNA and synthesize both ET-1 and ET-2 peptides under basal and stimulated conditions. ET-2 gene expression has been described in fetal human kidney [54], but this is the first demonstration of ET-2 gene expression by cells of adult human kidney.

Not only do cultured human proximal tubular cells synthesize endothelins, but they also express binding characteristics and mRNA for both endothelin receptor subtypes, that is, ET_A and ET_B. Although their functions have not been defined so far, ET_B receptors are the major receptor subtype expressed in the kidney [47, 48], mainly by tubular cells [55, 56]. In addition, rat proximal tubular cells appear to express a subpopulation of ET_A receptors *in vitro* [37] and *in vivo* [45], though this has been disputed [55]. Since we found a 6:1 ratio of ET_A to ET_B receptors, it seems likely that ET_B receptors are down-regulated *in vitro*.

Several lines of evidence suggest that ET-1 acting via ET_B receptors is an autocrine growth factor in human renal proximal tubular cells. Firstly, phosphoramidon, which reduces basal ET-1 production by inhibiting the ET-1 converting enzyme [57], decreased basal DNA synthesis. Secondly, blockade of ET_A and ET_B receptors (by PD-145065) reduced basal mitogenesis, but selective blockade of ET_A receptors (by BQ-123) did not. Thirdly, where seen, the mitogenic response to exogenous endothelins, that is, ET-1, ET-2 and ET-3 appeared to be roughly equipotent and could be blocked by PD-145065, indicating that this was mediated through the non-selective ET_B receptor subtype. These results



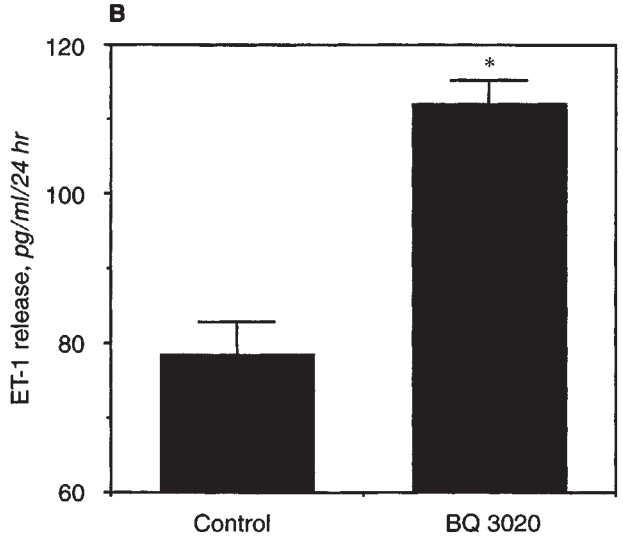


Fig. 8. *Tubular ET-1 synthesis (pg/ml) over 24 hours.* (**A**) Effect of phosphoramidon (Phos, 10^{-4} M), BQ-123 (10^{-6} M) and PD-145065 (PD, 10^{-5} M); $N = 9$ to 11 from four experiments; (**B**) the effect of BQ-3020 (10^{-7} M); $N = 10$ to 11 from three experiments. $^{*}P < 0.05$ vs. control.

show that, unlike rat mesangial cells [58] and rat vascular smooth muscle cells [39], ET_B receptors mediate the mitogenic response to endothelins in human tubular cells.

A surprising finding was that antagonism of the ET_B receptor by PD-145065 was associated with marked suppression of endogenous ET-1 synthesis. A likely explanation for this finding is that antagonism of the ET_B receptor interrupts a positive feedback loop by which endogenous ET-1 induces its own synthesis. Autoinduction of ET-1 synthesis via the ET_B receptor has been described in human endothelial cells [59]: the effect seen was small (20 to 40% increase) and comparable to the increase we noted with BQ-3020 (Fig. 8B). Given the 10-fold decrease seen with effective ET_B receptor blockade (PD-145065), the small magnitude of both these effects could reflect the already 'near maximal' stimulation of ET-1 synthesis by endogenous ET-1.

Since proximal tubular ET-1 synthesis *in vivo* is likely to be low, this phenomenon suggests a mechanism by which an increase in proximal tubular ET-1 gene expression (for example, by hypoxic induction) may contribute significantly towards the previously unexplained long-lasting induction of ET-1 gene expression in the post-ischemic kidney [21]. These findings thus point to a novel function for tubular ET_B receptors in mediating both mitogenesis and autoinduction of ET-1 synthesis, which differs from that of tubular ET_A receptors, which probably mediate sodium handling [45].

Recent studies have reported the beneficial effects of the selective ET_A receptor blocker, BQ-123 in initiating tubular regeneration in a rat model of severe acute renal failure, independent of its effect on glomerular filtration rate and renal plasma flow [45, 60]. This was a surprising finding since there has been some dispute as to whether rat tubular cells express ET_A receptors [37, 55]. Although the role of tubular ET_B receptors was not addressed in these studies, blockade of tubular ET_A receptors could have 'favored' a mitogenic response to tubular-derived ET-1 via tubular ET_B receptors. Together with our findings, this would imply a different pathophysiological role for each receptor subtype in ischemia such that potentially different and opposing effects, that is, 'dysfunction' (via ET_A) and 'regeneration' (via ET_B) could result from increased tubular ET-1 synthesis. Indeed, the severity of the ischemic insult may be the most important determinant of which effect predominates, since no effect of BQ-123 has been found in models of acute renal failure of lesser severity, where early tubular regeneration would be the norm [45].

The effect of hypoxia on tubular ET-1 gene expression was specific for this endothelin isoform, since ET-2 gene expression did not change and ET-3 gene expression was not induced. Although hypoxic induction of the ET-1 gene has been reported in endothelial cells [22], the effect of hypoxia on ET-2 and ET-3 gene expression in other cell types has not been previously described. Moreover, the hypoxic induction of tubular ET-1 gene expression is particularly significant since, unlike endothelial cells, we [24] and others [61] have found few agents that consistently up-regulate tubular ET-1 production. For example, we have previously found that only phorbol myristate acetate (PMA) and human high density lipoproteins (HDL) consistently increased tubular ET-1 mRNA levels and peptide synthesis [24]. Thus it seems likely that the regulation of the ET-1 gene differs between tubular epithelial cells and endothelial cells [24, 62, 63].

It has been reported that lesser degrees of hypoxia (10% O_2) *in vivo* increase ET-1 gene expression in the lung and cardiac atrium but not kidney [64]. This suggests that the renal threshold for hypoxic up-regulation of the ET-1 gene might be lower than in the other two organs. The mechanism by which ET-1 gene expression is increased by hypoxia in cultured human PTC is not known, but the fact that cobaltous chloride does not increase ET-1 mRNA levels suggests that it is different from that which regulates erythropoietin [33].

In summary, we have presented evidence that that human renal proximal tubular cells synthesize ET-1 and ET-2 constitutively and that ET-1, acting via ET_B receptors, is an autocrine growth

factor for these cells. In conjunction with the demonstration that the endothelins are mitogenic, the specific up-regulation of ET-1 gene expression by hypoxia, and the possibility that ET-1 can induce its own synthesis in these cells, provides a plausible mechanism whereby tubular ET-1, acting via ET_B receptors, may be involved in the regenerative response of surviving tubular cells to ischemic injury. Preliminary observations have also shown that hypoxia itself stimulates DNA synthesis in quiescent human tubular cells [65], and that this response is associated with specific down-regulation of ET_B receptor number (unpublished data). Further studies are being conducted as to the precise contribution of the ET-1/ET_B growth loop in this response, as well as the importance of this autoregenerative system *in vivo*.

Acknowledgments

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